

Berberine Inhibits the Production of Lysophosphatidylcholine-induced Reactive Oxygen Species and the ERK1/2 Pathway in Vascular Smooth Muscle Cells

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Lysophosphatidylcholine (lysoPC) induces vascular smooth muscle cell (VSMC) proliferation and migration, which has been proposed to initiate the intimal thickening in coronary atherosclerotic lesions. Berberine is an alkaloid in *Berberis aquifolium* and many other plants. Recently, it has been shown to have beneficial effects on the cardiovascular system, such as anti-hyperglycemic and cholesterol-lowering activity. In this study, we investigated its effects on lysoPC-induced VSMC proliferation and migration. Berberine inhibited lysoPC-induced DNA synthesis and cell proliferation in VSMCs, as well as migration of the lysoPC-stimulated VSMCs. It also inhibited the activation of extracellular signal-regulated kinases (ERKs) and reduced transcription factor AP-1 activity and the lysoPC-induced increases in intracellular reactive oxygen species (ROS). These results indicate that the inhibitory effects of berberine on lysoPC-stimulated VSMC proliferation and migration are attributable to inhibition of ROS generation and hence of activation of the ERK1/2 pathway. This suggests that berberine has potential in the prevention of atherosclerosis and restenosis.

Keywords: Berberine; ERK1/2; Lysophosphatidylcholine; Reactive Oxygen Species; Vascular Smooth Muscle Cells.

Introduction

Abnormal proliferation and migration of vascular smooth

muscle cells (VSMCs) play a critical role in the development of atherosclerosis and restenosis after percutaneous coronary angioplasty. Lysophosphatidylcholine (lysoPC), a major phospholipid component of low-density lipoproteins (LDL), stimulates the proliferation and migration of VSMCs (Kohn et al., 1998), and its concentration is higher in atherosclerotic arteries than in normal arteries (Yla-Herttuala et al., 1989). Recently, it was reported that lysoPC-induced ERK1/2 activation is mediated by reactive oxygen species (ROS) (Yamakawa et al., 2002). ROS play a key role in inducing VSMC proliferation, which is important in the pathogenesis of intimal thickening in atherosclerosis and restenosis (Chen et al., 2003). ROS generation has been shown to be related to the activation of mitogen-activated protein (MAP) kinases and transcription factors, such as activator protein-1 (AP-1) (Han et al., 2003; Martindale and Holbrook, 2002). LysoPC potentiates vascular contractile responses in the rat aorta by activating ERK (Suenaga and Kamata, 2003). Consequently, lysoPC-induced MAP kinase activation in VSMCs could be involved in the pathogenesis of vascular atherosclerosis. A number of studies have been performed to explore the effects of pharmacological inhibitors on VSMC proliferation and migration (Andres and Castro, 2003).

Berberine is an isoquinoline alkaloid that has been isolated from *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree turmeric), *Berberis vulgaris* (barberry), and *Hydrastis canadensis* (goldenseal). It has antibiotic (Stermitz et al., 2000), antitumor (Iizuka et al., 2003) and antidiarrheal (Choi et al., 2003) activities. It also appears to have effects on cardiovascular diseases, such as arrhythmia,

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Abbreviations: ERK, extracellular signal-regulated kinases; lysoPC, lysophosphatidylcholine; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

congestive heart failure, hypertension, and myocardial infarction (Lau *et al.*, 2001). It is, however, still unclear whether berberine directly inhibits lysoPC-induced VSMC proliferation and migration, and what mechanisms are involved in these responses *in vitro*. Since many cell proliferation factors, including various mitogens, initiate similar signaling events such as MAP kinase activation, we hypothesized that berberine might affect MAP kinase activation in response to lysoPC. In the present study, we tested this idea and sought to identify potential mechanisms underlying the effect.

Materials and Methods

Cell culture VSMCs were isolated as previously described (Gunter *et al.*, 1982). Briefly, aortas were enzymatically isolated from the thoraxes of 8 week-old Sprague-Dawley rats. They were transferred to a plastic tube containing 5 ml of the enzyme dissociation mixture and incubated for 2 h at 37°C. The suspension was centrifuged at $1,000 \times g$ for 10 min and the pellet resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin in 75-cm² flasks at 37°C in a humidified atmosphere of 95% air and 5% CO₂. All experiments were conducted using VSMCs between passages 7 and 15, after growth arrest by incubation in DMEM containing 0.5% FBS for 48 h.

[³H]-Thymidine incorporation To evaluate DNA synthesis, thymidine incorporation assays were performed. VSMCs were plated in 24-well plates at 2×10^4 cells/ml. The cells were maintained for 24 h in medium containing 0.5% serum, and then treated with lysoPC after incubation with or without berberine for 1 h. After 24 h, 2 µCi/ml of [³H]-thymidine was added for 2 h. The cells were then washed three times with ice-cold PBS, incubated for 30 min in 10% trichloroacetic acid on ice, washed twice in ice-cold 95% ethanol, and lysed in 0.2 N NaOH. Samples were measured by liquid scintillation counter. Data are expressed as percentages of the radioactivity measured under basal conditions.

MTT assay To measure cell viability and proliferation, we used the MTT assay employing 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma, USA). VSMCs were seeded in 24-well culture plates at a concentration of 2×10^4 cells/ml and treated with lysoPC after incubation with or without berberine for 1 h. MTT solution (in PBS) was added to each well to a final concentration of 0.5 mg/ml and incubation continued in a humidified atmosphere at 37°C for 4 h, to allow MTT reduction. The formazan salt crystals were dissolved by adding dimethylsulfoxide (DMSO) and absorbances were measured at 570 nm with a microtiter plate reader.

Cell migration assay VSMC migration was examined by a

modified Boyden chamber assay with a Transwell cell culture chamber (Corning, USA), which allows cells to migrate through an 8-µm pore-size polycarbonate membrane (Law *et al.*, 1996). Polycarbonate membranes with pores were coated with 10 µg/well of gelatin solution in DMEM containing 0.5% FBS, and dried. VSMCs were suspended in DMEM with 0.5% FBS, pre-treated with berberine for 1 h and seeded (2×10^4 cells/ml) into Transwell inserts. These were placed in a 24-well plate containing DMEM with 0.5% FBS, and migration was stimulated by adding lysoPC to the bottom well of the Boyden chamber. After 12 h, the surfaces of the upper membranes were swabbed with cotton-tipped applicators to remove non-migrating cells, and the inserts were fixed in methanol for 30 min and stained with 1% crystal violet for 2 h. For quantitation, the cells on the surface of the upper membrane were removed with methanol. This allowed quantitative optical density analysis using a microtiter plate reader.

Measurement of intracellular ROS Intracellular ROS were measured in 24-well plates using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable, oxidation-sensitive probe (Tarpey and Fridovich, 2001). The dye is cleaved and trapped intracellularly, where it can be oxidatively modified by intracellular ROS and peroxidases to produce fluorescent 2',7'-dichlorofluorescein (DCF). Quiescent VSMCs were treated with lysoPC for 7 min after exposure to different berberine concentrations for 30 min, and then incubated with DCFH-DA for 7 min. The medium was removed, the cells were washed twice with PBS, and samples resuspended in PBS were analyzed by flow cytometry at 488 nm excitation, 535 nm emission.

SDS-PAGE and Western blot analysis After appropriate treatment, cells were rinsed with cold PBS, scraped into 1.5 ml tubes and solubilized in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 1 µg/ml leupeptin), followed by centrifugation at $12,000 \times g$ for 20 min at 4°C. The protein concentrations in the supernatants were determined with a Bradford protein assay kit (Bio-Rad, USA). Cell lysates containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking in 10 mM Tris-Cl buffer (pH 8.0) containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, the membranes were treated with primary antibodies, followed by appropriate horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody bands were detected with an enhanced chemiluminescence kit (Amersham Bioscience, Sweden), and quantified by densitometry.

ERK1/2 activity assay ERK1/2 activity was assayed following the instructions with the commercial assay kit (Cell Signaling, USA). Briefly, cells were harvested with lysis buffer supplied by

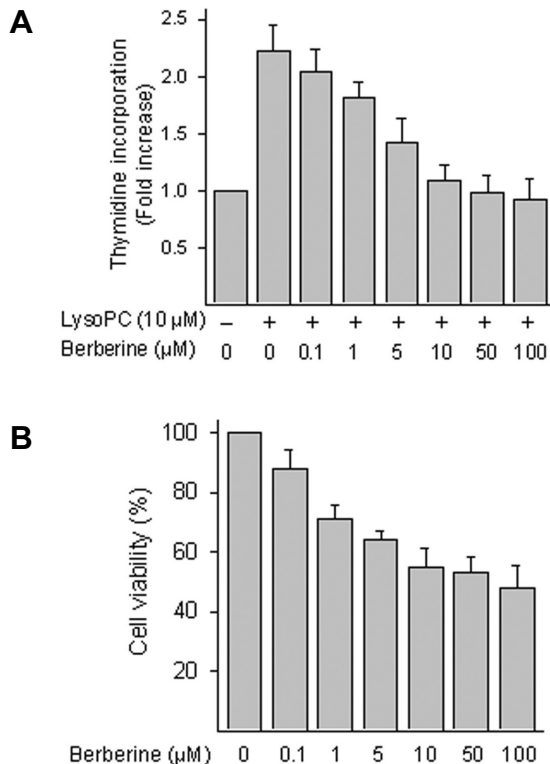


Fig. 1. Effect of berberine on lysoPC-induced DNA synthesis (A) and cell proliferation (B) in VSMCs. **A.** Quiescent cells were pretreated with different concentrations of berberine for 1 h. The cells were then stimulated with lysoPC (10 μ M) for 24 h with [3 H]-thymidine present for the final 2 h. **B.** Quiescent cells were pretreated with berberine for 1 h and incubated with 10 μ M of lysoPC for 48 h. Cell viability was quantified by the MTT assay. The results represent means \pm SEM of three replicate measurements in three separate experiments.

the manufacturers and centrifuged at $12,000 \times g$ for 10 min at 4°C . The cell lysates were incubated overnight at 4°C with 20 μ l of an immobilized dual phospho-specific ERK1/2 monoclonal antibody, and washed twice with lysis buffer and kinase buffer. The kinase reactions were performed in the presence of 200 μ M ATP and Elk-1 protein at 30°C for 20 min and terminated with 25 μ l of SDS-loading buffer. Phosphorylation of Elk-1 was measured by Western blot analysis using antibody against phospho-Elk-1 (Ser383) and the LumiGLO system supplied by the manufacturer.

Luciferase reporter assay VSMCs were plated on 35-mm dishes and transfected with a luciferase reporter construct possessing consensus AP-1 binding sites (pAP1-Luc) (BD Biosciences, USA). After incubation for 12 h, the medium was exchanged for fresh DMEM, and the cells were further incubated for 24 h. The VSMCs were then treated with lysoPC after incubation with or without berberine for 1 h, harvested and luciferase activities measured with a luciferase reporter assay kit (BD Biosciences, USA) and a luminescence spectrophotometer.

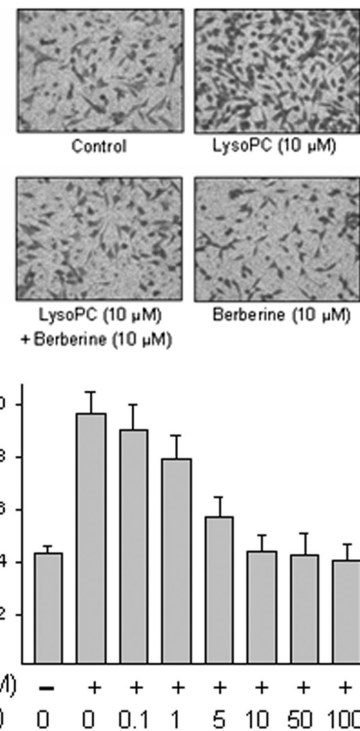


Fig. 2. Effect of berberine on lysoPC-induced VSMC migration. VSMCs were pretreated with 10 μ M of berberine for 1 h and seeded into Transwell inserts that were placed in a 24-well plate. LysoPC (10 μ M) was added to the bottom of each well. After 12 h, cells that migrated to the other side of the membrane were stained with 1% crystal violet and eluted with methanol, and quantitative analyses were performed by optical density measurement using a microtiter plate reader. The results represent the means \pm SEM of three different experiments.

Specific luciferase activities were normalized for transfection efficiency using the corresponding β -galactosidase activities, and expressed as activities relative to the control.

Statistical analysis Data are expressed as means \pm S.E.M. Statistical comparisons were performed by one-way analysis of variance and Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Inhibitory effect of berberine on lysoPC-induced cell proliferation We examined the effect of berberine on lysoPC-induced DNA synthesis in VSMCs, by measuring [3 H]-thymidine incorporation. 24 h of lysoPC treatment (10 μ M) increased DNA synthesis about 2.2-fold (Fig. 1A), and pretreatment of the cells with berberine (0.1–100 μ M) inhibited this lysoPC-induced DNA synthesis in a concentration-dependent manner (Fig. 1A). Berberine also inhibited lysoPC-induced proliferation of VSMCs

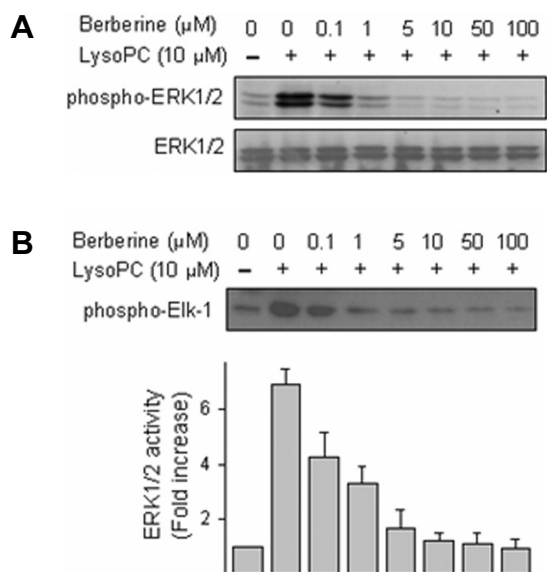


Fig. 3. Effect of berberine on activation of ERK1/2 by lysoPC. Quiescent VSMCs were treated with different concentration of berberine for 1 h and stimulated with lysoPC (10 μM) for 15 min. **A.** Equal amounts of protein were separated by SDS-PAGE and analyzed by Western blotting. Phosphorylation of ERK1/2 was detected using a phospho-specific ERK1/2 antibody. Blots are representative of three experiments, all with similar results. **B.** Cells were lysed and equal amounts of extracted protein were immunoprecipitated with phospho-specific ERK1/2 antibody. Samples were then incubated with an Elk-1 protein in the presence of ATP. Phosphorylation of Elk-1 was measured by Western blotting using phospho-Elk-1 (Ser383) antibody. Signals were quantified by scanning densitometry and the graph shows relative activities. The results represent the means \pm SEM of three different experiments.

(Fig. 1B). Since DNA synthesis was reduced to approximately the control level by 10 μM berberine, we used this concentration in subsequent experiments.

Effect of berberine on lysoPC-induced cell migration

The effect of berberine on lysoPC-induced VSMC migration was examined with a modified Boyden chamber assay. Treatment of VSMCs for 12 h with lysoPC induced a nearly 2-fold increase in the number of migrating cells and berberine inhibited this effect (Fig. 2).

Effect of berberine on lysoPC-induced ERK1/2 activation

We tested whether berberine inhibited the phosphorylation of ERK1/2 (Yamakawa *et al.*, 2002) in lysoPC-stimulated VSMCs. ERK1/2 phosphorylation increased in response to 15 min incubation with lysoPC after serum starvation, and pretreatment with berberine was inhibitory (Fig. 3A). The levels of ERK1/2 protein were not affected by the treatment with berberine. We also tested whether the inhibition of ERK1/2 phosphorylation by berberine

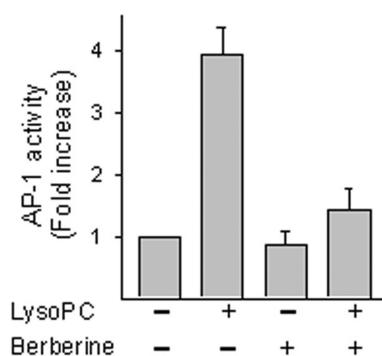


Fig. 4. Effect of berberine on AP-1 activity induced by lysoPC. VSMCs transfected with pAP1-Luc were incubated for 24 h with lysoPC in the presence or absence of berberine (10 μM). Luciferase activity is expressed as activity relative to the untreated group (control). The results represent the means \pm SEM of three different experiments.

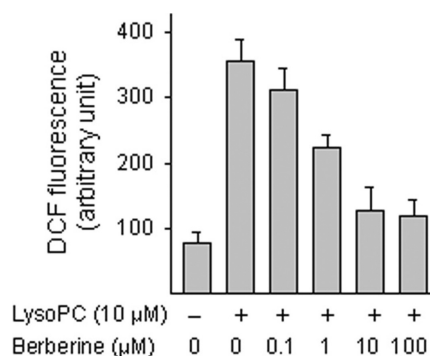


Fig. 5. Effect of berberine on intracellular ROS formation induced by lysoPC. After incubation with or without berberine for 30 min, VSMCs were stimulated with 10 μM of lysoPC for 7 min. Results are representative of at least three independent experiments with similar results.

was paralleled by an effect on ERK1/2 activity. ERK1/2 activity was analyzed by immunoprecipitation with an immobilized ERK1/2 antibody followed by *in vitro* kinase assay with an Elk-1 fusion protein as substrate for ERK1/2. As shown in Fig. 3B, lysoPC stimulated ERK1/2 activity and this effect was inhibited by pretreatment with berberine in a manner paralleling the results of the Western blot analysis. Since ERK1/2 activation stimulates AP-1 activity (Kim and Iwao, 2003) we assessed the effect of berberine on lysoPC-induced AP-1 activity by means of a reporter assay (see **Materials and Methods**). Berberine pretreatment inhibited the increase in AP-1-mediated reporter activity induced by lysoPC (Fig. 4).

Inhibitory effect of berberine on intracellular ROS generation

Stimulation of vascular cells with lysoPC causes an increase in ROS (Kugiyama *et al.*, 1999; Yamakawa *et al.*, 2002). We tested whether berberine pre-

vented lysoPC-induced ROS generation. Figure 5 shows that berberine reduced the formation of ROS in response to lysoPC.

Discussion

Proliferation of VSMCs has been implicated as one cause of atherosclerosis and post-intervention restenosis (Cho *et al.*, 2005; Rivard and Andres, 2000). Hence many therapeutic approaches attempt to inhibit VSMC proliferation and migration (Andres and Castro, 2003). Oxidized LDL plays a pivotal role in the initiation and progression of atherosclerosis by affecting the activities of VSMCs, endothelial cells and monocytes. LysoPC, an active component of oxidized LDL, induces VSMC proliferation and migration (Kohn *et al.*, 1998, and see above). We have demonstrated that berberine, a natural product, inhibits this VSMC proliferation and migration. LysoPC is mitogenic and can activate ERK1/2 (Yamakawa *et al.*, 1998) and regulate contraction of VSMCs (Della Rocca *et al.*, 1997). ERK1/2, is a member of the serine/threonine kinase family, which is activated by a variety of stimuli involved in cell growth, as well as by endogenously generated ROS in VSMCs stimulated with lysoPC (Yamakawa *et al.*, 2002).

Recent studies have demonstrated that statin treatment can inhibit the proliferation and migration of VSMCs (Takemoto and Liao, 2001). In addition, pitavastatin suppresses lysoPC-induced VSMC proliferation by inactivating the ERK1/2 pathway (Yamakawa *et al.*, 2003). These results provide further support for the beneficial effect of cholesterol-lowering drugs, such as statins, on arterial walls. Recently Kong *et al.* identified berberine as a novel cholesterol-lowering drug with an action mechanism, i.e. increasing hepatic LDL receptors, different from that of statins (Kong *et al.*, 2004). Berberine is known to have antibiotic, antitumor, and anti-hyperglycemic activities. However, it was not known whether berberine inhibited cell proliferation, migration and ERK1/2 activation in VSMCs. In our study, berberine inhibited lysoPC-induced VSMC proliferation and migration in a dose-dependent manner. Moreover, it also reduced the activation of ERK1/2 and AP-1 by lysoPC. Therefore, it appears that berberine inhibits the proliferation and migration of VSMCs by suppressing the ERK1/2-AP-1 pathways.

ROS play a key role in VSMC proliferation, which is important in the pathogenesis of atherosclerosis, hypertension and restenosis after percutaneous coronary intervention (Chen *et al.*, 2003). Stimulation of VSMCs with lysoPC causes an increase in intracellular ROS and activates ERK1/2 (Yamakawa *et al.*, 2002). ERK1/2 activation by lysoPC is inhibited by antioxidants such as *N*-acetylcysteine, reduced glutathione monoester and vitamin E (Watanabe *et al.*, 2002; Yamakawa *et al.*, 2002).

Moreover berberine decreased ROS levels and inhibited angiogenesis in embryoid bodies, indicating that the compound was acting as a scavenger of intracellular free radicals (Wartenberg *et al.*, 2003). Our data confirm that berberine reduces intracellular ROS formation. It is, therefore, possible that the inhibitory effects of berberine on VSMC proliferation and migration result from its ability to inhibit activation of ERK1/2 by reducing ROS production in response to lysoPC.

Interestingly, clinical trials and animal studies have demonstrated that berberine prevents congestive heart failure (Zeng *et al.*, 2003), cardiac hypertrophy (Hong *et al.*, 2002) and arrhythmia (Lau *et al.*, 2001). It also inhibits COX-2 expression by inhibiting AP-1, a key transcription factor in inflammation and carcinogenesis (Kuo *et al.*, 2004). Moreover, in an animal model, berberine was also shown to have vasodilator (Ko *et al.*, 2000) and hypotensive (Kang *et al.*, 2002) effects, based on its modulation of K⁺ channel activity and inhibition of angiotensin-converting enzyme activity, respectively. Indeed berberine may have multiple effects on the cardiovascular system. These include decreasing plasma cholesterol, reducing vascular inflammation, enhancing endothelial function, and inhibiting VSMC proliferation. Its antiproliferative effect on VSMCs may contribute to its long-term beneficial effects on vascular walls, and its inhibitory action on MAP kinases, whose activation is dependent on intracellular ROS, may play an important role in the direct cellular effects of berberine on vascular walls.

In conclusion, our results demonstrate that berberine inhibits stimulation of VSMC proliferation and migration by lysoPC. Furthermore, the antiproliferative effect of berberine is mediated by inhibition of the ERK1/2 pathway, and may be attributed to blocking the production of ROS. These findings help to clarify the clinical benefits of berberine and provide a scientific rationale for its use in cardiovascular diseases such as atherosclerosis and arterial restenosis.

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